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ORIGINAL CONTRIBUTION

V. Tang
C. Torregrosa
X. Remesar
M. Alemany

Dietary oleoyl-estrone delays the growth rate of young rats

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V. Tang · C. Torregrosa · X. Remesar · Marià Alemany (✉)
Centre Especial de Recerca en Nutrició i Ciència dels Aliments
Departament de Bioquímica i Biologia Molecular,
Facultat de Biologia, Universitat de Barcelona
Av. Diagonal, 645
08 028 Barcelona, Spain
e-mail: alemany@bio.ub.es

Summary *Background* Oleoyl-estrone administration induces the rapid loss of fat preserving body protein. *Aim of the study* We intended to check whether the fat-shedding effect of oleoyl-estrone arrests growth in young rats, limiting the buildup of protein and fat. *Methods* Oleoyl-estrone diluted in a powdered hyperlipidic diet (33 μmol/kg) was given for 30 days to 30-day old Zucker lean (Fa/?) rats. Their body weight and food consumption were followed daily; on day 30 of treatment (60-day old rats), whole body composition (lipid, protein) was determined, and plasma energy parameters and leptin were measured. *Results* Oleoyl-estrone-treated rats grew more slowly than controls fed the hyperlipidic diet alone, and on day 60 their lipid content was about half that of controls. Protein content per kg was identical in both groups, but treated rats tended to accumulate less nitrogen and energy because of their smaller size. No changes in plasma glucose, urea, triacylglycerols or total cholesterol were observed, but oleoyl-estrone-treated rats showed lower circulating leptin than controls. *Conclusion* Despite limiting the accumulation of lipids, oleoyl-estrone slowed, but did not arrest growth of young rats, nor elicit a loss of fat or protein.

Key words growth – oleoyl-estrone – nitrogen balance – energy balance – leptin

Introduction

Oleoyl-estrone induces a dose-dependent loss of body fat reserves when chronically injected to adult rats (1,2), reducing the body fat content, sparing protein (1,2) and maintaining plasma metabolite levels within the physiological range in spite of the massive mobilization of fat reserves (1). Oleoyl-estrone acts by inducing a decrease in food intake with unchanged – or at least less decreased – energy expenditure, sustained mostly by internal fat stores (2). Under these conditions, glucose availability is maintained, with increased liver glycogen and higher sensitivity to insulin, the levels of which decrease in lean and obese rats (3,4). The increased mobilization of lipids results in diminished body weight and lowered rates of tissue accrual, but no significant losses of protein occur (1,2,5,6). This contrasts with the mobilization of fat found during acute starvation, when body protein supplies most of the hydrocarbon skeletons for the synthesis of glucose (7). Chronic starvation or restrictive diets, supplying a similar proportion of energy as that voluntarily ingested by oleoyl-estrone treated rats, result in diminished energy expenditure (8) and the establishment of mechanisms that protect tissue fat and protein (9). In the treatment with oleoyl-estrone, these effects are not observed, and "normality" is the rule in all circulating energy metabolism indicators (1,3,5,6).

Oleoyl-estrone decreases insulin and leptin levels, eliciting a marked glucocorticoid response (3). Chronic treatment with oleoyl-estrone decreases the expression of the *Ob* gene (3) in lean but not in *fa/fa* rats (4), and

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lowers the ponderostat reference setting in lean but – again – not in *fa/fa* rats (5). Leptin is a key factor for the development of sexual differentiation (10), affecting growth and the metabolic environment during adolescence (11).

The ample availability of glucose, and low amino acid catabolism, together with increased availability of fatty acids as a consequence of massive lipolysis mimic – with respect to amino acid metabolism – the situation found in rats receiving a high-energy diet such as the "cafeteria" diets (12). Under cafeteria-diet conditions, however, growth is enhanced and there is a marked lipid deposition (13); but amino acid oxidation is limited, increasing the size of the "nitrogen gap" (14), i. e. the mass of nitrogen lost via unknown pathways and unaccounted for in nitrogen balances (15).

The high growth rates of young weaned rats and the efficient deposition of protein and, to a lesser extent, of fat and other body components are maintained by increased food intake (16) and high-energy diets (17). Since oleoyl-estrone treatment tends to diminish appetite and promote the oxidation of fat stores sparing protein, we have devised an experiment in which the drive to accrue body components, enhanced by a high-energy diet, is counteracted by the oleoyl-estrone-induced wasting. In this way we can examine whether oleoyl-estrone hinders growth and the accrual of living matter during postnatal development.

Materials and methods

Two groups of 30-day-old female Zucker lean (*Fa/?*) rats from Charles River (Gannet, France) stock, initially weighing 65–75 g were used. They were kept under standard conditions in metabolic cages (Techniplast Gazzada, Guggugiate, Italy). The animals were fed with a powdered hyperlipidic diet (B&K, Sant Vicent dels Horts, Spain) that contained 22.18 % fat, 17.23 % protein, 4.93 % fiber, 1.86 % mineral and vitamin concentrate (Bestmix 821105, Special Diet Service, B&K), 36.31 % starch and 3.43 % sugars, with a gross energy content (bomb calorimeter, C–7000, IKA, Heitersheim, Germany) of 19.79 MJ/kg, and metabolizable energy of 16.97 MJ/kg; 46.6 % of the energy was derived from lipids, 37.2 % from carbohydrates and 16.1 % from protein. Samples of HL diet were used for the evaluation of naturally occurring acyl-estrone esters by extraction with anhydrous methanol in a Soxhlet followed by saponification and RIA analysis of the estrone released (18). The diet contained a mean 1.23 ± 0.39 (N=5):mol/kg of fatty-acyl esters of estrone. A part of this diet was supplemented at the source with oleoyl-estrone (Salvat, Esplugues de Llobregat, Spain), the analysis of the supplemented diet showed a 33.3 ± 3.0:mol/kg (N=5) content in acyl-estrone (diet HL-OE). Each diet (i. e., HL used as control, and HL-OE) was fed for 30 days to a group of 6 rats. The weights and food consumption of the rats were measured daily; urine and droppings were also recovered, weighed and stored. The dose of oleoyl-estrone ingested by the rats was calculated from the food intake and acyl-estrone content of the diet.

At the end of the experiment, the rats were anesthetized with ethyl ether, and blood was taken via heart puncture and used to obtain plasma. The rats were then killed and dissected, cleaned of intestinal contents, weighed again and sealed in polyethylene bags that were subsequently autoclaved at 120 °C for 2 hours; the whole rat was then minced to a smooth paste with a blender.

Plasma was used for the estimation of glucose, total cholesterol, total protein, triacylglycerols and urea using a dry-chemistry strip auto analyzer (Spotchem, Menarini, Firenze, Italy), as well as for the estimation of leptin (RL83K kit, Linco Research, St Charles, MO, USA).

The rat body paste was used for the estimation of the proportions of lipids (19), energy (bomb calorimeter) and nitrogen, the latter measured as total N with a Carlo Erba (Milano, Italy) NA–1500 elemental analyzer, and then converted to protein using a factor of 5.5 (20). The nitrogen content of the diet, droppings, and urine were also measured and used for the estimation of nitrogen balance (21).

The amount of energy accrued (E_A, expressed in kJ) in 30 days was estimated using the equation

$$E_A = BW_{60} \cdot e_F - BW_{30} \cdot e_C$$

where BW_{30} and BW_{60} are the body weights (in g) on day 30 and at the end of the experiment, respectively; e_C is the energy equivalence found for HL rats on day 60, and e_F is the energy equivalence found for the corresponding group (HL or HL-OE) on day 60 (in kJ/g of carcass). Energy expenditure (E_E) was calculated as the difference between the metabolizable energy in the food ingested (E_I) and the energy accrued

$$E_E = E_I - E_A$$

the energy balance components were expressed in W (J/s) calculated as the mean for the 30-day period studied. Since the final weights of both experimental groups were different, an allometric correction factor of body weight ($BW^{0.75}$) (22) was used to render the E_E data comparable.

Nitrogen accrual (N_A, expressed in g of N) was estimated from the equation

$$N_A = BW_{60} \cdot n_F - BW_{30} \cdot n_{HL}$$

where n_{HL} is the N concentration in HL rats on day 60, and n_F the N concentration of the corresponding group (HL or HL-OE) on day 60 (in mgN/g of carcass). The extent of the "nitrogen gap" (21) was estimated as the difference between the sum of N accrued and excreted

through urine and stool and the amount of N ingested with the food.

Statistically significant differences ($P < 0.05$) between groups were determined using the Student's *t* test.

Results

Fig. 1 shows the changes in body weight observed in 30-day old rats fed the hyperlipidic diet with or without added oleoyl-estrone. HL rats grew at a high and constant rate (Table 1), practically doubling that of rats receiving oleoyl-estrone (HL-OE). The food consumption (Fig. 2) was the same in both groups, in spite of the lower final body size of the HL-OE rats. No differences were found between groups when comparing the cumulative food ingested during 30 days. Although HL and HL-OE rats ingested a similar amount of water and food per day, the HL-OE rats produced less fecal matter and excreted more urine than the HL group (Table 1).

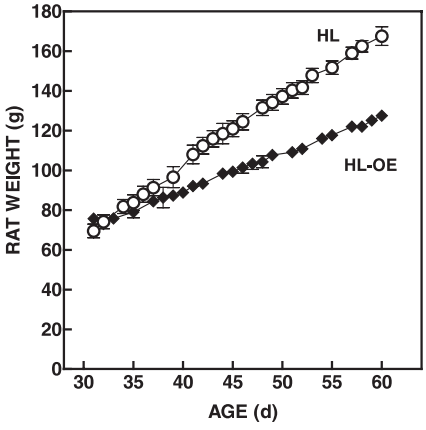

Fig. 1 Body weight changes in young lean Zucker rats treated for 30 days with the HL and HL-OE diets. Each point represents the mean ± SEM of 6 different animals per group.

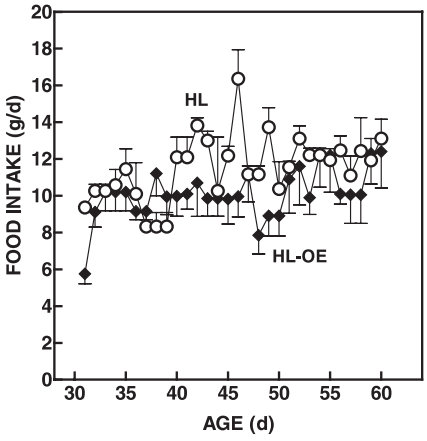

Fig. 2 Daily food intake of young lean Zucker rats treated for 30 days with the HL and HL-OE diets. Each point represents the mean ± SEM of 6 different animals per group.

The composition of the rat carcasses is presented in Table 2. There were no differences between groups in protein or in energy content per unit of rat weight, but the larger-size HL rats tended to contain more energy and protein than the oleoyl-estrone-treated rats. Lipid content, however, was lower in HL-OE rats both per unit of body weight and in the total body content, which was about half that of HL.

Fig. 3 presents the energy and nitrogen balances of both groups of rats. The energy accrued by HL-OE rats was 55 % of that of HL, but energy expenditure was 94 %. When the energy expenditure data were corrected by an allometric factor, the values for both groups were indistinguishable: 53.5 $mW/g^{0.75}$ (HL) and 56.6 $mW/g^{0.75}$ (HL-OE). The net efficiency of deposition of energy (i. e. the ratio of accrued energy/energy expenditure) of HL rats was higher (0.18 ± 0.02) than that of the HL-OE (0.11 ± 0.01; $P < 0.05$), which means that untreated rats had a higher efficiency (i. e. the percentage of ingested energy accrued) in the use of the ingested energy: 15.2 ± 1.1 % versus 9.6 ± 1.3 % in the HL-OE rats ($P < 0.05$).

Nitrogen accrual was also higher in HL than in HL-OE rats: 27.9 % and 19.1 % of the N ingested, respectively. The amount of nitrogen lost in urine and stool

Tab. 1 Weights, intake and excretion parameters of 30-day old lean Zucker rats treated with the HL and HL-OE diets for 30 days

Parameter	Units	HL	P	HL-OE
Initial weight (day 30) BW_{30}	g	69.5 ± 3.6	ns	73.7 ± 2.4
Final weight (day 60) BW_{60}	g	167.0 ± 5.0	*	128.1 ± 2.6
Growth rate	g/d	2.97 ± 0.07	*	1.65 ± 0.07
Water intake	mL/d	13.9 ± 1.0	ns	16.1 ± 0.9
Food ingested	g/d	11.51 ± 0.99	ns	10.13 ± 0.82
Stool produced (wet weight)	g/d	2.59 ± 0.06	*	1.80 ± 0.07
Urine excreted	mL/d	4.46 ± 0.34	*	7.40 ± 0.76
Oleoyl-estrone intake[1]	µmol/kg · d	0.12 ± 0.02	*	3.34 ± 0.29

The values are the mean ± SEM of 6 animals per group; * = $P < 0.05$.
[1] Calculated using as body weight the mean between days 30 and 60.

Tab. 2 Whole body composition of 30-day old lean Zucker rats treated with the HL and HL-OE diets for 30 days

Parameter	Units	HL	P	HL-OE
Protein (Nx5.55) content	%	19.10 ± 0.44	ns	19.81 ± 0.36
	g	31.9		25.3
Lipid content	%	10.60 ± 0.73	*	7.33 ± 0.66
	g	17.7		9.4
Total energy content	kJ/g	9.16 ± 0.21	ns	9.14 ± 0.32
	MJ	1.53		1.17

The values are the mean ± SEM of 6 animals per group; * = $P < 0.05$.

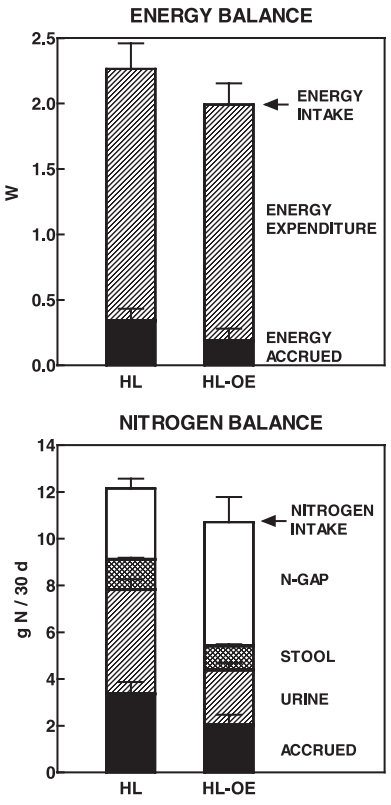

Fig. 3 Energy and nitrogen balances of young lean Zucker rats treated for 30 days with the HL and HL-OE diets. In all cases, the total height of the column corresponds to the energy/nitrogen intake. The experimental (i. e. measured) data are presented as the mean ± SEM of 6 animals. All other data are given as a mean value, calculated as indicated under Materials and Methods. The amount of nitrogen excreted in urine and stool of OE-HL rats was significantly ($P < 0.05$) lower than in HL group.

was also lower under oleoyl-estrone administration, giving rise to a nitrogen gap that was 75 % higher than that of HL controls.

Table 3 shows the plasma levels of some metabolites and leptin of both groups studied. There were no statistically significant differences in glucose, cholesterol, urea, triacylglycerols or total protein between HL and HL-OE rats. The latter, however, showed leptin levels about 1/3 of those of HL.

Tab. 3 Plasma metabolites and leptin of 30-day old lean Zucker rats treated with the HL and HL-OE diets for 30 days

Plasma parameter	Units	HL	P	HL-OE
Glucose	mM	9.50 ± 0.31	ns	8.61 ± 0.41
Total cholesterol	mM	1.63 ± 0.15	ns	1.85 ± 0.06
Urea	mM	10.40 ± 0.62	ns	9.03 ± 0.64
Triacylglycerols	mM	0.97 ± 0.11	ns	0.75 ± 0.11
Total protein	g/L	51.6 ± 2.55	ns	56.8 ± 1.97
Leptin	nM	0.38 ± 0.05	*	0.12 ± 0.01

The values are the mean ± SEM of 6 animals per group; * = $P < 0.05$.

Discussion

The standard dose of oleoyl-estrone used in most i. v. studies is 3.5 μmol/kg^{-d} (1,3), practically the same as that ingested by the treated rats in this study. The young rats, however, in spite of receiving a dose that induces a significant loss of body weight and fat in adult rats, maintained a steady growth rate, with unchanged metabolic parameters. Treated rats maintained the same rates of energy expenditure per comparable unit of body weight, and their food intake was not diminished (especially when their smaller size is taken into account). The differences between groups were found only in the rates of accrual of energy and overall growth.

Hyperlipidic cafeteria diets induce a marked increase in the growth rates of young rats (17). The rates found here are in the range of 2.5 % of body weight per day, and contrast with the – also high, but lower – rate of treated rats: 1.6 % of body weight per day. The main factor affected by the treatment with oleoyl-estrone was the lipid content. The relative reduction of fat is in agreement with the induction of fat utilization observed in adult rats treated with oleoyl-estrone (1,6). If we apply the same approach described in the Materials and methods for the estimation of energy accrual to lipid accrual, the result of 30 days of treatment is an increase in lipids of about 20 %, which contrasts with the accrual of protein, about 70 % of the initial stock. In HL, both components increased by 140 %. The accrual of lipid in treated rats was minimal, which agrees with the well-known fat-depleting effects of oleoyl-estrone (1,2).

Oleoyl-estrone induces a decrease in energy intake in adult rats, maintaining their energy expenditure, thus creating an energy gap filled with the energy stored in adipose tissue (1,2); this effect was not observed in the young rats studied here. The 30–60 day animals are growing at a fast pace, and a significant part of their energy goes into tissue buildup, in contrast with the adult rats, in which there is no net body mass growth and in which the energy intake matches energy expenditure. This differential shift into the fate of available energy may hamper the appetite-suppressing effects of oleoyl-estrone, since growth is the main energetic priority over fat accumulation. Oleoyl-estrone affected more significantly fat accumulation than growth. In fact, the small difference in energy intake of the HL-OE versus HL rats closely matched the differences in energy accrued (mainly lipid), as shown in Fig. 3. These data are consequent with oleoyl-estrone acting on the control of the mass of lipid reserves (5), but not hampering growth.

Other effects of oleoyl-estrone were observed in the diminished leptin levels, a consequence of the inhibition of the *Ob* gene expression by oleoyl-estrone (3), which may have some bearing on the low rates of accrual shown by treated animals, since leptin is known to promote development during adolescence (11). The prob-

lem remains, however, to determine the actual extent of the effect of this inhibition on the circulating leptin, since the smaller mass of lipids (and hence of adipose tissue) in HL-OE rats should also result in lower leptin synthesis rates; the lower circulating leptin of the oleoyl-estrone-treated rats may thus be explained by a combination of both factors.

Oleoyl-estrone induced the same effects on the mass of lipids in young as in adult rat, since the percentage of lipids in the body of young HL-OE rats was lower than in the HL group. Thus, oleoyl-estrone indeed reduced the fat content of the oleoyl-estrone-treated animals; the HL-OE rats were smaller, but they had an even lower lipid amount than the HL group.

The lower stool excretion of oleoyl-estrone-treated rats may be a consequence of altered water balance, which agrees with the higher production of urine (and similar water intake). This may be due to the catecholamine-like actions of oleoyl-estrone, such as the direct induction of lipolysis in isolated adipocytes (23); catecholamines both stimulate urine excretion and slow intestinal peristalsis, increasing water reabsorption and thus diminishing the mass of droppings. The lower loss of fecal nitrogen of treated rats gives support to the hypothesis that oleoyl-estrone slows intestinal peristalsis.

The nitrogen balance of oleoyl-estrone-treated rats shows altered nitrogen handling, similar to that observed in cafeteria diet-fed rats (14), in which the wide availability of energy and glucose induces the sparing of protein, which is freed for growth (24). This is due in part to an inhibition of the urea cycle (25), which limits the removal of amino nitrogen. When the urea cycle operation is hampered, a significant part of the nitrogen is lost through a pathway as yet unknown (21), probably in the form of gas (15). The pattern of distribution of the nitrogen balance components agrees with this conjecture.

The main point drawn from this study is, however, that oleoyl-estrone does not counteract growth; at most it may hinder the deposition of some body components and slow growth, especifically fat deposition. In spite of showing other effects, the dose given is not sufficient to arrest growth nor to elicit the loss of fat reserves; in any case, the treated animals show a fairly low proportion of lipids, but there was no loss of fat, only an arrest of its accretion.

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